Accuracy of Klason Lignin and Acid Detergent Lignin Methods As Assessed by Bomb Calorimetry†

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An accurate method for estimation of lignin concentration is important for prediction of the digestible energy content of livestock feeds. The accuracy of lignin concentration estimates based on the Klason lignin and acid detergent lignin methods was compared. Ten diverse forage samples were analyzed for protein, carbohydrates, lipids, organic acids, ash, lignin (by both methods), and gross energy. The accuracy of the two lignin concentration estimates was examined by comparing the measured forage gross energy to a gross energy value calculated from the compositional analysis. Use of the acid detergent lignin estimate in this gross energy calculation accounted for 68−84% of the forage gross energy compared to 85−97% of the gross energy using Klason lignin. These results indicate that while Klason lignin estimates are substantially higher than acid detergent lignin estimates, Klason lignin is the more accurate lignin method and does not overestimate lignin because gross energy recoveries were less than 100%.

Keywords: *Klason lignin; acid detergent lignin; forages; calorimetry*

INTRODUCTION

Lignin is a cell-wall polymer derived from free-radical reactions of hydroxycinnamyl alcohols and other cell-wall constituents (Boudet et al., 1995). Lignin has been implicated as the primary component of cell walls that limits forage cell-wall digestibility by ruminants (Jung and Deetz, 1993). A major difficulty in studying the role of lignin in cell-wall digestibility has been that a definitive molecular structure cannot be drawn for lignin and all lignin concentration estimates are purely empirical, based on the particular method of analysis chosen. As a result, lignin concentration estimates vary widely among methods. While Klason lignin is the standard method of analysis for wood (Lai and Sarkanen, 1971; Kirk and Obst, 1988), use of this method for forages has been questioned because of possible contamination with protein (Lai and Sarkanen, 1971; Van Soest, 1967). The acid detergent lignin (ADL) method was developed as an alternative to Klason lignin (Van Soest, 1967) and is the most commonly used method for lignin determination in animal science and agronomy.

It has previously been shown that Klason lignin, which gives lignin concentration estimates for forages 2−5 times higher than does ADL, is not significantly contaminated with protein or carbohydrate and is similar to ADL in molecular composition (Hatfield et al., 1994). Data also indicate that the ADL method underestimates lignin concentration due to loss of acid-soluble lignin in the acid detergent step of the procedure (Kondo et al., 1987; Lowry et al., 1994). However, to date, no research has presented quantitative data comparing the accuracy of these methods for estimating lignin concentration in forages.

Our objective was to compare the accuracy of the Klason lignin and ADL methods for measuring lignin concentration in forages by employing an independent analytical method. We reasoned that by determining the chemical composition of forages, and using bomb calorimetry to measure gross energy, it should be possible to compare the accuracy of the two lignin methods by determining how much of the sample's gross energy could be accounted for by calculation of gross energy based on compositional data. Calorimetry data are presented in this report, verifying that ADL does not account for all of the plant lignin and Klason lignin does not overestimate lignin concentration.

MATERIALS AND METHODS

A diverse group of 10 forage samples was used in this study including alfalfa (*Medicago sativa* L.), Kura clover (*Trifolium ambiguum* Beib.), annual medic (*Medicago* spp.), maize (*Zea mays* L.), orchardgrass (*Dactylis glomerata* L.), smooth bromegrass (*Bromus inermis* Leyss), switchgrass (*Panicum virgatum* L.), and oat (*Avena sativa* L.). All forages were dried at 60 °C and ground to pass a 1-mm screen in a cyclone mill. Samples were analyzed for crude protein (N × 6.25) by macro-Kjeldhal (AOAC, 1990). Total carbohydrates were determined by a two-stage acid hydrolysis with neutral sugars quantified by GC (Theander and Westerlund, 1986) and uronic acids by colorimetry (Ahmed and Labavitch, 1977). Lipids were determined by exhaustive extraction with ether (AOAC, 1990). Organic acids were determined by HPLC (Weimer et al., 1991)

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Table 1. Composition of Forage Samples (% DM[b])

sample	crude protein	carbohydrate	lipid	organic acids			ash	lignin	
				acetate	lactate	malate		ADL[a]	Klason
alfalfa	21.3	44.5	1.8	1.1	nd[c]	2.4	8.8	7.1	15.0
alfalfa	16.2	46.1	2.3	1.3	nd	3.4	10.6	6.6	13.6
Kura clover	21.1	41.2	4.0	0.8	nd	3.8	8.9	2.9	8.4
annual medic	25.9	38.5	2.9	0.9	nd	7.8	14.3	3.6	9.3
maize silage	7.1	63.3	3.6	1.1	2.2	nd	5.0	2.4	12.2
maize silage	11.1	59.6	4.2	1.4	3.6	nd	7.5	2.5	10.0
orchardgrass	14.2	42.9	4.4	0.7	nd	nd	11.4	1.9	12.6
smooth bromegrass	11.6	52.9	1.6	1.5	nd	nd	9.0	4.2	15.5
switchgrass	9.3	51.2	1.3	1.4	nd	1.0	8.3	3.0	14.2
oat straw	4.2	60.4	0.6	1.0	nd	nd	10.7	4.6	16.6

[a] Acid detergent lignin. [b] Dry matter. [c] Not detected.

analysis of the extract obtained by a 30 min extraction of 500 mg of the forage samples with 6 mL of 0.1 M NaOH, and centrifugation at 12000*g* for 10 min. The ash content of all samples was determined by combustion at 450 °C. Klason lignin was determined as the ash-free residue from the two-stage H_2SO_4 hydrolysis (Theander and Westerlund, 1986; Hatfield et al., 1994). Acid detergent lignin was determined by sequential detergent analysis (Van Soest and Robertson, 1980). All compositional data were determined in duplicate and are reported on a dry matter (DM) basis.

Bomb calorimetry (Model 1241 Adiabatic Calorimeter, Parr Instrument Co., Moline, IL) was used to determine the gross energy of forage samples. A calculated gross energy value for the forage samples was determined by applying gross energy values for the forage components to the measured concentrations of those components. Gross energy contents for most of the components were taken from Brody (1945). However, gross energy contents were determined for malate and four lignin preparations. The lignins included commercially available alkali, organosolv, and hydrolytic lignins (Aldrich Chemical Co., Inc., Milwaukee, WI) and a dehydrogenation polymer (DHP) lignin synthesized from coniferyl alcohol (Kirk and Brunow, 1988). The lignins also were analyzed for carbohydrate and ash contamination, as described above, and for lignin composition by pyrolysis−GC−MS analysis to calculate a syringyl-to-guaiacyl (S/G) ratio (Ralph and Hatfield, 1991; Jung and Buxton, 1994).

The gross energy content of the forage samples was calculated by equation I. The calculations were done using both the ADL and Klason lignin concentration estimates. Calculated

$$\text{gross energy} = (\text{protein} \times 5700 \text{ kcal kg}^{-1}) + (\text{carbohydrate} \times 4000 \text{ kcal kg}^{-1}) + (\text{lipid} \times 9400 \text{ kcal kg}^{-1}) + (\text{acetate} \times 3500 \text{ kcal kg}^{-1}) + (\text{lactate} \times 3700 \text{ kcal kg}^{-1}) + (\text{malate} \times 2739 \text{ kcal kg}^{-1}) + (\text{lignin} \times 6957 \text{ kcal kg}^{-1}) \quad \text{(I)}$$

gross energy values were compared to the corresponding measured gross energy of the forages.

RESULTS AND DISCUSSION

The composition of the forage samples is presented in Table 1. The major chemical components varied widely among samples: from 4.2 to 25.9% for crude protein, 38.5 to 63.3% for carbohydrate, 0.6 to 4.4% for lipid, and 5.0 to 14.3% for ash. Small quantities of acetate were present in all forage samples. Significant quantities (1.0−7.8%) of malate were found in the legumes and in switchgrass. Only trace amounts of succinate were found (<0.15% of DM), and the other organic acids associated with the tricarboxylic acid cycle were not detected. The maize silage samples contained significant amounts of lactate produced by the fermentation during ensiling. As expected, lignin concentration estimates were very different for the ADL and Klason lignin methods (overall means of 3.9 ± 0.6 and 12.7 ± 0.9% DM, respectively). The ADL and Klason lignin concentration estimates were positively correlated (r = 0.65, $P < 0.05$), as has been observed previously (Hatfield et al., 1994; Jung et al., 1997).

Table 2. Composition of a Dehydrogenation Polymer Lignin (DHP) and Three Isolated Lignins

sample	% DM[c]				S/G[b] (normalized units)	gross energy (kcal kg^{-1} DM[c])
	carbohydrate	ash	lignin ADL[a]	lignin Klason		
DHP	0.6	0.3	25.9	88.6	0	6981
alkali	2.3	6.1	21.4	90.1	0.07	6360
organosolv	0.9	0	33.8	83.7	2.47	6652
hydrolytic	3.2	1.1	10.6	83.4	1.26	6501

[a] Acid detergent lignin. [b] Syringyl-to-guaiacyl ratio. [c] Dry matter.

The compositions of the isolated lignins and the DHP lignin are given in Table 2. The gross energy of the four lignins ranged from 6360 to 6981 kcal kg^{-1} DM. These values are similar to the value of 6371 kcal kg^{-1} DM reported for Klason lignin prepared from Douglas fir (*Pseudotsuga menzeisii* Mirb.) wood (Shafizadeh and DeGroot, 1976). When analyzed for Klason lignin content, the four lignins ranged in concentration from 83.4 to 90.1%. All lignins were quite soluble in the ADL procedure, with ADL concentration estimates of only 10.6−33.8%. The alkali lignin was apparently isolated from gymnosperms (softwoods) as the S/G ratio was 0.07, whereas the organosolv and hydrolytic lignins must have been isolated from angiosperms (hardwoods) because the S/G ratios were 2.47 and 1.26, respectively. The lignins contained small amounts of ash and carbohydrate contaminants. Recovery of DM by compositional analysis ranged from 84.6 to 98.7%. For calculation of the caloric density of lignin, we assumed all DM that was not accounted for by analysis was acid-soluble lignin. After correction of the gross energy determinations for the isolated lignins based on ash and carbohydrate contaminants, a mean value of 6957 kcal kg^{-1} lignin was calculated.

Gross energy concentration of the forages averaged 4389 ± 32 kcal kg^{-1} (Table 3) and was in agreement with other reported values (Brody, 1945). The percentage of measured gross energy that could be accounted for by calculation from the composition of the forage samples ranged from 67.6 to 96.6% (Table 3). The two lignin methods resulted in very different percentages of gross energy that could be accounted for by compositional analysis (77.3 ± 1.8 and 91.4 ± 1.3% for ADL and Klason lignin, respectively). Obviously, the difference in calculated gross energy was due entirely to the

Table 3. Measured Gross Energy Concentration of Forage Samples and Percentage of Gross Energy Accounted for by Calculation from Composition

sample	measured gross energy (kcal kg^{-1} DM)[b]	calculated gross energy (%)	
		ADL[a]	Klason lignin
alfalfa	4493	82.3	94.5
alfalfa	4371	79.8	90.9
Kura clover	4460	77.5	86.1
annual medic	4308	82.9	92.1
corn silage	4392	81.1	96.6
corn silage	4497	83.8	95.4
orchardgrass	4485	69.0	85.6
smooth bromegrass	4323	75.7	93.9
switchgrass	4377	67.6	85.4
oat straw	4182	73.3	93.3

[a] Acid detergent lignin. [b] Dry matter.

difference between the two lignin estimates as all other forage component concentrations were identical between calculations. If Klason lignin overestimates lignin concentration in forages, as suggested by Van Soest (1967) and others (Lai and Sarkanen, 1971), then it would be expected that the calculated gross energy based on the Klason lignin value would result in a higher gross energy than actually determined for the sample. This was not the case for any forage sample and supports the conclusion of Hatfield et al. (1994) that Klason lignin is a more accurate measure of forage lignin concentration. The fact that calculation of forage gross energy using the ADL estimate resulted in underestimation of actual sample gross energy by approximately 23% would be expected from the suggestion that lignin is solublized and lost in the ADL method (Kondo et al., 1987; Lowry et al., 1994). The high solubility of the isolated lignins used in our study when analyzed for ADL supports these previous reports.

A concern that must be expressed relative to the data presented above is that only 92.7 ± 1.3% of the total forage DM was accounted for by chemical composition analysis when the Klason lignin estimate was included. Dry matter recovery was, of course, significantly lower (83.8 ± 1.8%) when lignin was estimated by ADL. Similar incomplete recoveries of forage DM by compositional analysis have been reported. For artificially dried orchardgrass, perennial ryegrass (*Lolium perenne* L.), and timothy (*Phleum pratense* L.), Waite et al. (1964) reported DM recoveries ranging from 92.0 to 95.9% using a modification of the lignin method described by Ellis et al. (1946). This lignin method is similar to the ADL method as the sample is treated with dilute acid (0.8 M H_2SO_4) at high temperature (100 °C) prior to hydrolysis with 12 M H_2SO_4 at low temperature (20 °C). Waite et al. (1964) reported lignin concentrations of 2.7−9.4% for the grasses sampled. Goto et al. (1991) reported DM recoveries for sorghum (*Sorghum bicolor* Moench × *Sorghum sudanese* Stapf) of between 77.4 and 84.1% using the acetyl bromide method (Morrison, 1972), a method in which lignin is solubilized and measured spectrometrically. They also reported DM recoveries for the same forage samples of 80.6−87.7% using the Christian lignin method (Christian, 1971), which is similar to the Klason lignin method because the 12 M H_2SO_4 treatment (25 °C) precedes the dilute acid hydrolysis at high temperature (1.1 M H_2SO_4, 100 °C). Lignin concentration estimates ranged from 4.2 to 8.0% for the acetyl bromide method and 2.6 to 11.8% for the Christian lignin values (Goto et al., 1991). Ternrud and Neergaard (1986) used the Klason lignin method and found DM recoveries of 92.6−96.6% in barley (*Hordeum vulgare* L.). No comparable data on the proportion of DM that can be accounted for by compositional analysis using the ADL method have been found.

When the gross energy that was not accounted for by compositional analysis was divided by the amount of DM not accounted for by analysis, it was found that the missing DM should have a gross energy content of 4901 ± 485 kcal kg^{-1} for the Klason lignin method and 6012 ± 180 kcal kg^{-1} for the ADL procedure. Data for the annual medic sample were excluded from the preceding calculation because the predicted caloric density of the missing DM was greater than any known organic constituent of plants using either of the lignin estimates. The reason for this outcome with the annual medic sample is not known. The predicted gross energy content for the ∼7% of DM unaccounted for in the Klason lignin calculation would indicate the missing forage components should primarily be nonlignin constituents such as protein and carbohydrate. In contrast, the much larger caloric density predicted for missing DM when ADL (∼16%) is used indicates that more energy-dense components, such as lipid or lignin, also must have been underestimated in the component analysis.

The results using compositional analysis and bomb calorimetry support the conclusion of Hatfield et al. (1994) that Klason lignin is a more accurate measure of forage lignin concentration than is the more commonly used ADL procedure. While these two lignin methods can both be used to predict forage digestibility with similar accuracy (Jung et al., 1997), it is important to have accurate estimates of forage lignin concentration when formulating animal diets. This is because lignin has no digestible energy value for animals and if lignin concentration is underestimated, then the missing organic matter is erroneously assumed to be carbohydrate or other feed components having nutritional value to the animal.

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